

Award Number: W81XWH-12-1-0014

TITLE: Regulation of ATM-Dependent DNA Damage Responses in Breast Cancer by the RhoGEF Net1

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REPORT DATE: May 2015

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE May 2015		2. REPORT TYPE Final		3. DATES COVERED 01-Apr-2014– 31- May-2015	
4. TITLE AND SUBTITLE Regulation of ATM-Dependent DNA Damage Responses in Breast Cancer by the RhoGEF Net1				5a. CONTRACT NUMBER W81XWH-12-1-0014	
				5b. GRANT NUMBER	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Wonkyung Oh, Ph.D. E-Mail: wonkyung_oh@uth.tmc.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Texas Health Science Center 7000 Fannin st. FL2, Houston, TX77030-5400				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT The Neuroepithelioma transforming gene 1 (Net1) is a RhoA specific guanine nucleotide exchange factor (GEF) that is frequently overexpressed in human cancer, including breast cancer. We have previously reported that DNA damage activates Net1 to control RhoA and p38 MAPK mediated cell survival pathway in response to ionizing radiation (IR). However, others have shown that Net1 activation contributes to RhoB-mediated cell death after IR. Thus, the role of Net1 in controlling IR responses and cell survival is controversial. With the completion of the first year of this fellowship, we have found that the Net1A isoform is specifically required for DNA double-strand break (DSB)-induced signaling and DNA repair. Depletion of Net1A in human breast cancer cells reduced IR-stimulated ATM activation and signaling to its substrates Chk2 and H2AX. In addition, suppression of Net1A expression adversely affected cell survival after IR. Moreover, we observed that overexpression of the Net1A isoform significantly reduced γH2AX foci formation after IR, which required the unique N-terminal region of Net1A. Importantly, this effect did not require Rho GTPase activation by Net1A, and was not recapitulated by overexpression of RhoA or RhoB. Net1A was also found to co-immunoprecipitate with the DNA-PK complex in an IR-regulated manner. Taken together, our current data suggests a model in which Net1A functions as a non-catalytic binding protein to control DNA damage response signaling and DNA repair to affect cell survival after IR.					
15. SUBJECT TERMS Net1, RhoGEF, Ionizing radiation, DNA damage response, DNA repair					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 13	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)

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Introduction

In the United States, breast cancer is the second most frequently diagnosed cancer in women (1). Ionizing radiation (IR) is commonly used to treat breast cancer patients after surgery, as well as for care of inoperable metastatic tumors (2). However, despite advances in the use of radiation therapy, a significant percentage of patients experience relapse of their cancers. Exposure to ionizing radiation (IR) causes double strand DNA breaks that are lethal to a cell if not repaired. In response to IR exposure a signal transduction cascade is initiated that activates cell cycle checkpoints to cause cell cycle arrest, thereby allowing cells time to repair their damaged DNA (3). The ability of a cell to efficiently respond to IR treatment and evade cell death forms the basis for radiation resistance in cancer cells to allow for tumor recurrence. Thus, understanding the mechanisms controlling DNA damage signaling is necessary to devise more efficient treatments for breast cancer.

Rho GTPases control diverse aspects of cell behavior, including organization of the actin cytoskeleton, cell migration, cell cycle progression, and gene expression (4). Rho GTPases acts as molecular switches by cycling between an active GTP-bound and an inactive-GDP bound state (5). The activation of Rho GTPases is regulated by specific guanine nucleotide exchange factors (GEFs) (6). Net1 (Neuroepithelial transforming gene 1) is a RhoA subfamily specific GEF that was originally identified as a transforming gene in NIH3T3 cell focus formation assays (7). Upregulation of Rho protein activity has been shown to contribute to tumor initiation and progression, and aberrant regulation of Rho proteins has been found in tumor cells (8, 9). Recently, we have shown that Net1 is overexpressed in human breast carcinomas and its co-expression with the $\alpha 6\beta 4$ integrin is associated with a high risk for distant metastasis in node positive breast cancer patients (10, 11, 12, 13). Previous work in our lab demonstrated that expression of the RhoA activating protein Net1 is required for cell survival following exposure to IR (14). Importantly, depletion of Net1 expression suppressed RhoA activation and induced cell death upon DNA damage (14). These results suggest that Net1 plays a critical role in the cellular response to DNA damage, and indicate that Net1 may be an attractive therapeutic target for sensitization of breast cancer cells to IR. Therefore, we decided to investigate the role of Net1 in DNA damage response signaling in breast cancer.

Body

Task 1: Determine the role of Net1 in ATM-mediated DNA repair (Months 24-27).

In the previous reporting period of the fellowship, we observed that knockdown of Net1A dramatically decreased activation of ATM and its downstream targets Chk2 and H2AX in MCF7 cells following IR treatment. We also showed that overexpressed Net1A suppresses ATM activation and phosphorylation of its downstream substrate H2AX in breast cancer in response to IR.

In response to DNA damage, mammalian cells have to arrest the cell cycle to allow time for DNA repair. Double strand breaks are repaired by two main pathways, including non-homologous end joining (NHEJ) and homologous recombination (HR) (15). ATM plays an important role in HR. For HR, double strand breaks are detected and processed by the MRN complex. This leads to recruitment and activation of ATM. ATM then phosphorylates many substrates required for arrest and repair. Because of its pivotal role in IR responses, ATM represents a critical target of breast cancer therapy and the downregulation of ATM has been implicated as a therapeutic strategy in radiosensitization of breast cancer (16, 17, 18, 19, 20). In addition, low levels of ATM expression is associated with advanced breast cancer and the loss of γ H2AX after IR is related to the rate of DSB repair (17).

We originally planned to measure DNA repair using DR-GFP DNA repair reporters in MCF7 cells, however, we had trouble to detect a DNA repair after establishment of the MCF7 stable cell lines. Alternatively, we examined the rate of DNA damage repair using neutral comet assay. The neutral comet assay (single-cell gel electrophoresis) is a sensitive and rapid technique for quantifying and analyzing DNA damage

in individual cells. We transfected MCF7 cells with control or Net1 isoform specific siRNAs and performed neutral comet assay after 4h recovery upon IR. We observed that loss of Net1A expression resulted in an increase in the amount of damaged DNA in both non-irradiated cells, as well as in cells four hours after irradiation (Figure 1A-C). These data suggested that Net1A expression is required for efficient DSB repair in breast cancer cells.

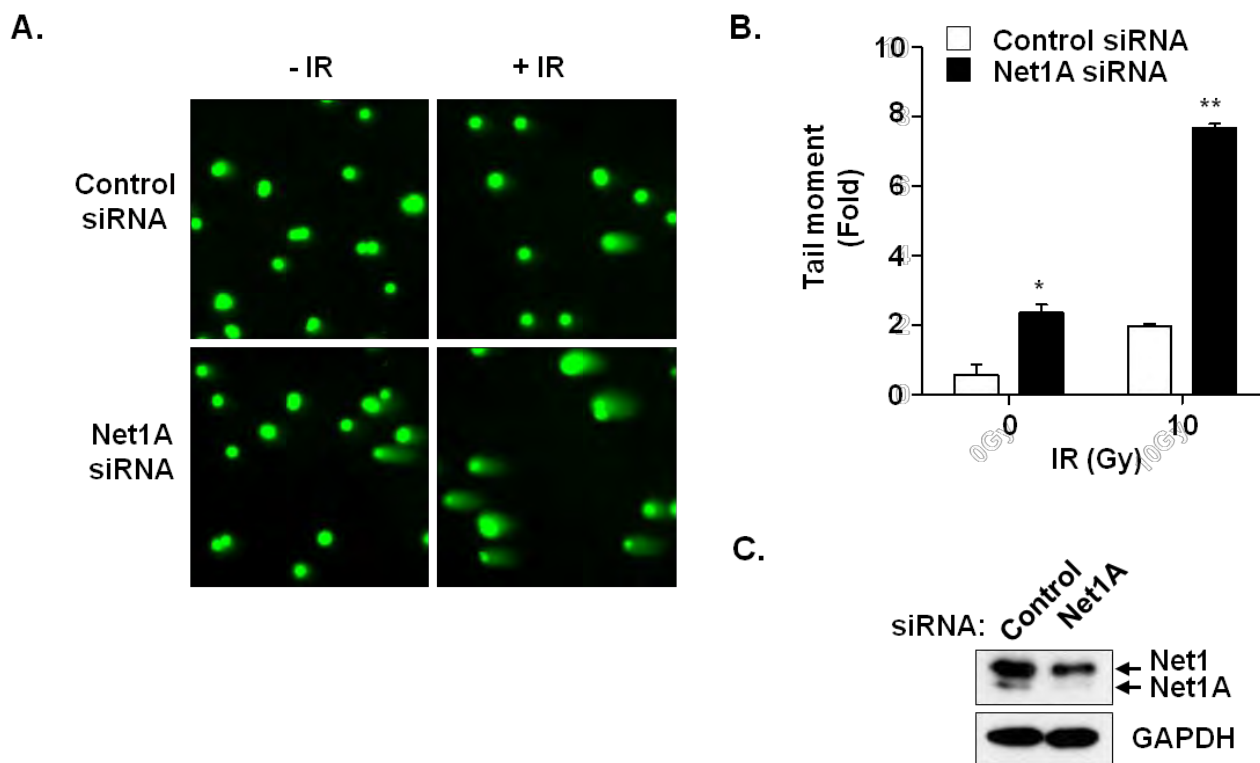


Figure 1. Effect of Net1A depletion in the recruitment ATM downstream substrates to DSBs. (A) Net1A knockdown delays DNA repair after IR. MCF7 cells were transfected with control or Net1A siRNAs. Three days later the cells were treated with 10 Gy IR. Four h later the cells were tested for DNA damage by comet assay. Shown is a representative experiment from three independent experiments. (B) Quantification of comet assays. Results are the average of three independent experiments. At least 100 cells were analyzed per sample. Errors are SEM; * = $p < 0.05$, ** = $p < 0.005$. (C) Representative Western blot of siRNA-transfected cells.

In our first year of this fellowship, we examined whether Net1A associates with ATM or DNA-PK in response to IR using a co-immunoprecipitation (coIP) assay. We found that Net1A did not interact with ATM in MCF7 cells in the presence or absence of IR. We also tested whether Net1A associates with DNA-PK complex following IR. DNA-PK consists of a catalytic subunit (DNA-PKcs) and a DNA-end binding component, the Ku70/80 heterodimer (21). We observed that Net1 interacts with DNA-PK and Ku70/80, and this interaction was enhanced following IR treatment and that N-terminus of Net1A is required for this interaction.

As a second assay, we conducted random plasmid integration assays to test whether Net1A regulates NHEJ repair following IR. MCF7 cells were transfected with control or Net1A specific siRNAs, and then re-transfected with linearized DNA that included a puromycin selection marker. Chromosomal integration of linearized DNA is dependent upon NHEJ activity, so selection for puromycin resistant cells provides a measure of the relative level of NHEJ activity. We observed that Net1A knockdown increased NHEJ efficiency, suggesting that NET1A inhibits NHEJ in the absence of DNA damage (Figure 2A-C).

Taken together, these data indicate that Net1A controls DNA repair in the presence or absence of DNA damage in breast cancer.

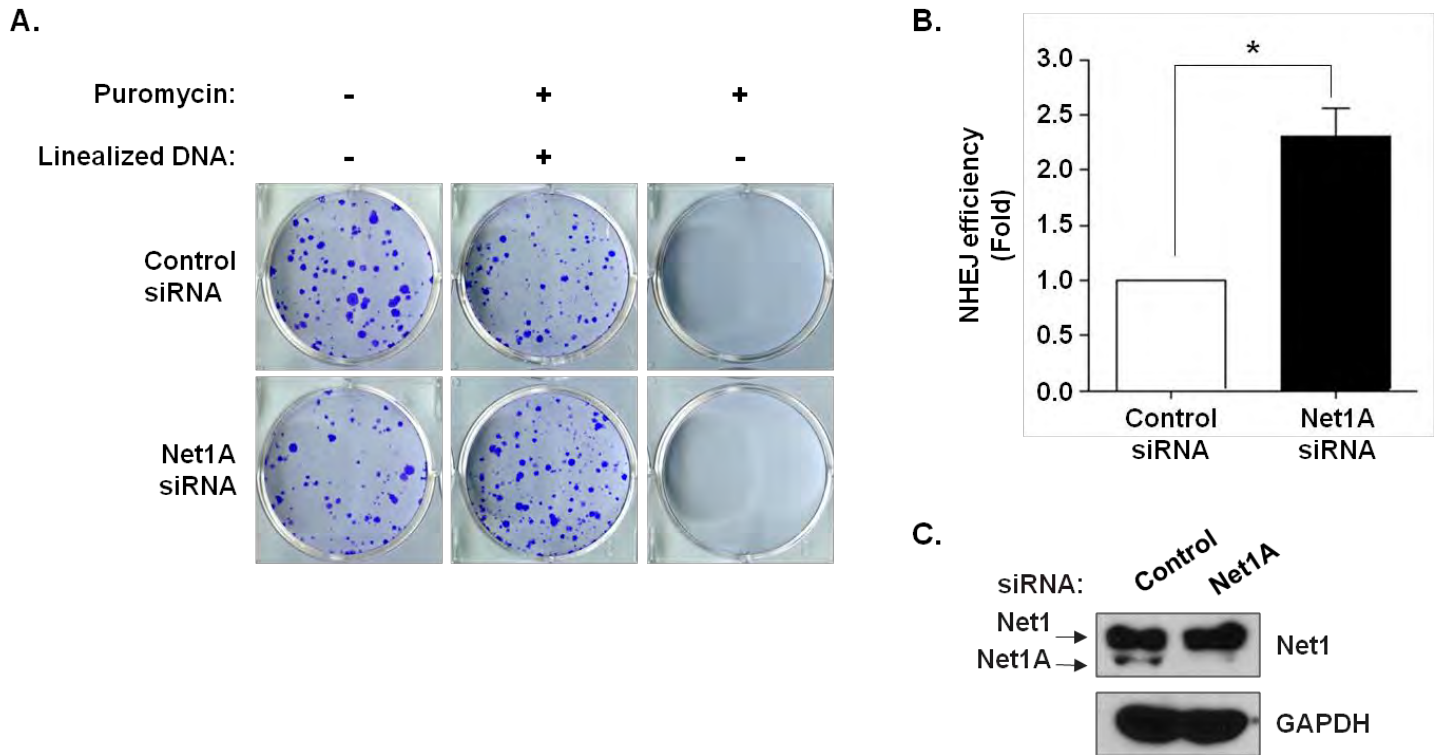


Figure 2. Net1A knockdown promotes NHEJ repair in the absence of DNA damage. (A) MCF7 cells were transfected with control or Net1A specific siRNAs. Two days later the cells were re-transfected linearized pBabe-puro and supercoiled EGFP expression plasmids. Integration of pBabe-puro requires NHEJ mediated repair. The next day the cells were replated and assessed for colony formation in the presence of puromycin. (B) Quantification of the survival fraction, which reflects NHEJ efficiency. Shown is the mean from 3 independent experiments. Errors are SEM. * = $p < 0.05$. (C) Representative Western blot of siRNA transfected cells. sample. Errors are SEM; * = $p < 0.05$, ** = $p < 0.005$. (C) Representative Western blot of siRNA-transfected cells.

Task 2: Determine the role of Net1 in normal breast epithelial cells responses to IR (Months 28-36).

Task 2a: Use MCF 10A and MCF12A cell lines for checkpoint kinase activation and IRIF formation to examine the significant difference between normal breast epithelial cells and cancer cells.

In year 1, we showed that depletion of Net1 inhibited phosphorylation of ATM and its downstream substrates Chk2 and H2AX in MCF7 breast cancer cells following IR treatment. We also found that transient expression of Net1A strongly suppressed IR-induced foci formation in breast cancer cells after IR. Furthermore, expression of catalytically-inactive form of Net1A (L267E) also suppressed γ H2AX staining, suggesting that the ability of Net1A to activate RhoA or RhoB was not required. Similarly, expression of a constitutively active form of RhoA (L⁶³RhoA) did not affect IR-induced foci formation.

To extend our studies on the role of Net1 regulation of DNA damage responses, we examined whether there are significant differences between normal breast epithelial cells and breast cancer cells. We used two different normal breast epithelial cell lines, MCF10A and MCF12A. We knocked down both Net1 isoforms by siRNA transfection and then we tested for activation of ATM, Chk2 and H2AX after IR exposure. We also

tested for effects on clonogenic survival. This approach allowed us to determine whether non-transformed breast epithelial cells also require Net1 expression for response to IR-induced DNA damage. Understanding of the role of Net1 in ATM-dependent DNA damage response between normal breast epithelial breast and cancer cells will contribute to develop potential new therapeutic targets for breast cancer patients.

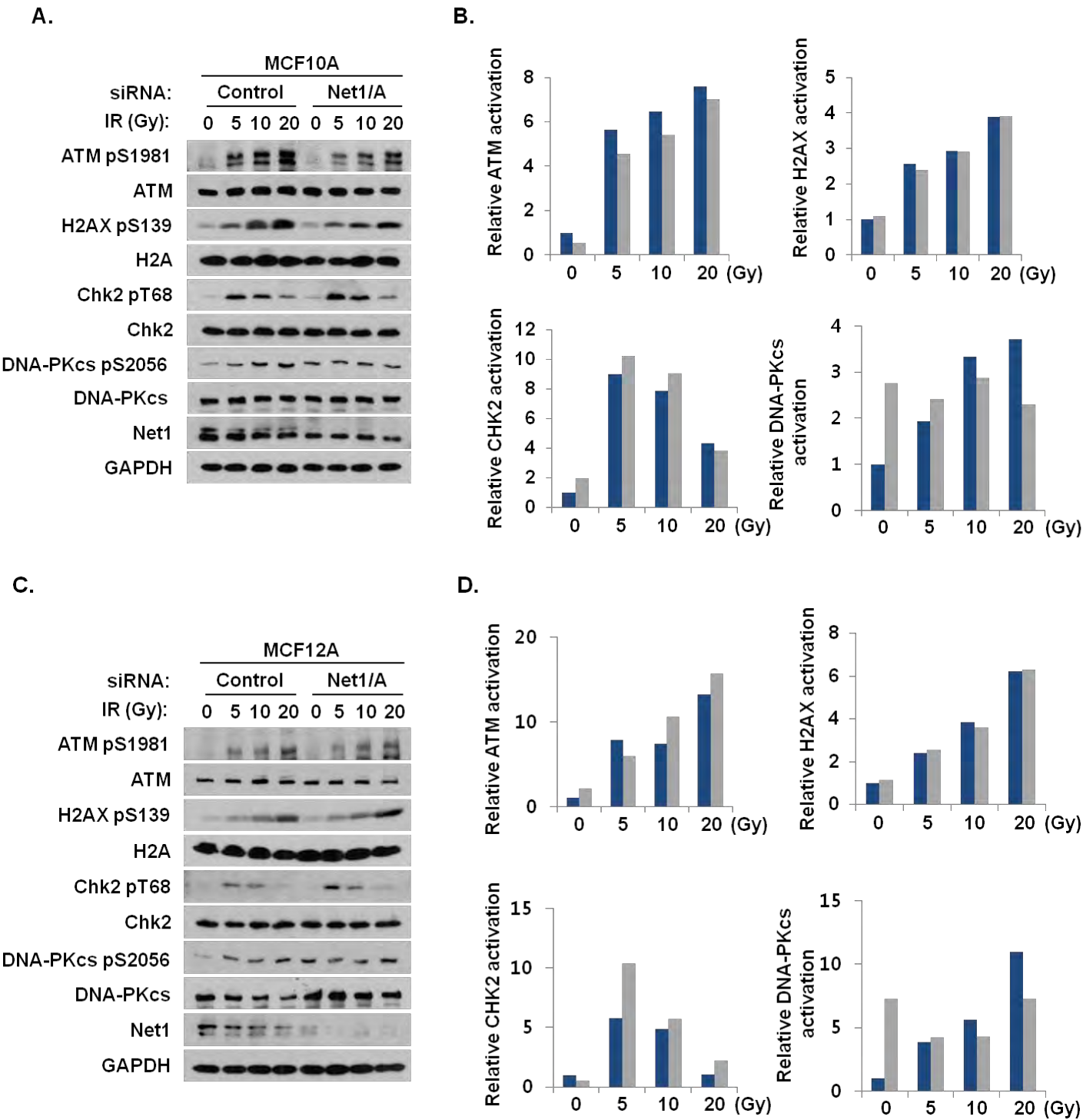


Figure 3. Net1 does not required for efficient ATM-dependent DNA damage signaling in normal breast cancer cells. (A) MCF10A cells were transfected with control siRNA or an siRNA specific for both Net1 isoforms. The cells were then treated with different amounts IR and harvested after 30 minutes. Phosphorylation or expression of the denoted proteins was detected by Western blotting. (B) Quantification of phosphorylation of ATM and its substrates upon Net1 knockdown. Phosphorylation was normalized to total ATM, Chk2, and H2AX. (C) MCF12A cells were transfected with control siRNA or an siRNA specific for both Net1 isoforms. The cells were then treated with different amounts IR and harvested after 30 minutes. Phosphorylation or expression of the denoted proteins was detected by Western blotting. (D) Quantification of phosphorylation of ATM and its substrates upon Net1 knockdown. Phosphorylation was normalized to

We observed that knockdown of Net1A did not significantly change activation of ATM and DNA-PKcs or their downstream targets Chk2 and H2AX following IR treatment (Figure 3A-D). Interestingly, we found that knockdown of Net1 potentiated phosphorylation of DNA-PKcs in the absence of IR, consistent with the results of our random plasmid integration assays (Figure 2) in MCF7 cells. Thus, these results indicate that Net1 is not critical for the ATM-dependent DNA damage response following IR exposure in normal epithelial breast cells, but may repress DNA-PK mediated NHEJ activity.

We also proposed to use MCF 10A and HMEC 184 cell lines for IRIF formation assays to examine the significant difference between normal breast epithelial cells and cancer cells. However, we did not see significant effects on IR-mediated ATM signaling and cell survival (Figure 4) in Net1 depleted normal epithelial breast cells. Thus, we stopped doing IRIF formation in normal breast epithelial cells.

Task 2b: Use MCF 10A and MCF12A cell lines for clonogenic survival assay to examine the significant difference between normal breast epithelial cells and cancer cells.

Our previous studies have shown that inhibition of Net1 expression sensitizes cells to the apoptotic effect of double strand DNA damage (14). Thus, this study indicates that Net1 regulates cell survival in response to double strand DNA damage in cancer cells. In year 2, we tested the functional importance of Net1A in cell survival following IR exposure in MCF7 cells using clonogenic survival assays (22, 23).. We observed that knockdown of Net1A reduced the number of colonies after IR. This study indicated that Net1A is required for MCF7 cell survival after exposure to IR.

In task 2b, we determined whether Net1A might be required for IR-induced cell death in normal breast epithelial cells. MCF12A cells were transfected with control siRNA or an siRNA specific for both Net1 isoforms and then treated with different doses of IR. The cells were then plated and allowed to form colonies for two weeks. We observed that knockdown of Net1 isoforms did not affect the number of colonies after IR (Figure 4A-C).

We conclude that Net1 isoforms are not required for ATM-mediated DNA damage signaling and cell survival in normal epithelial cells following IR.

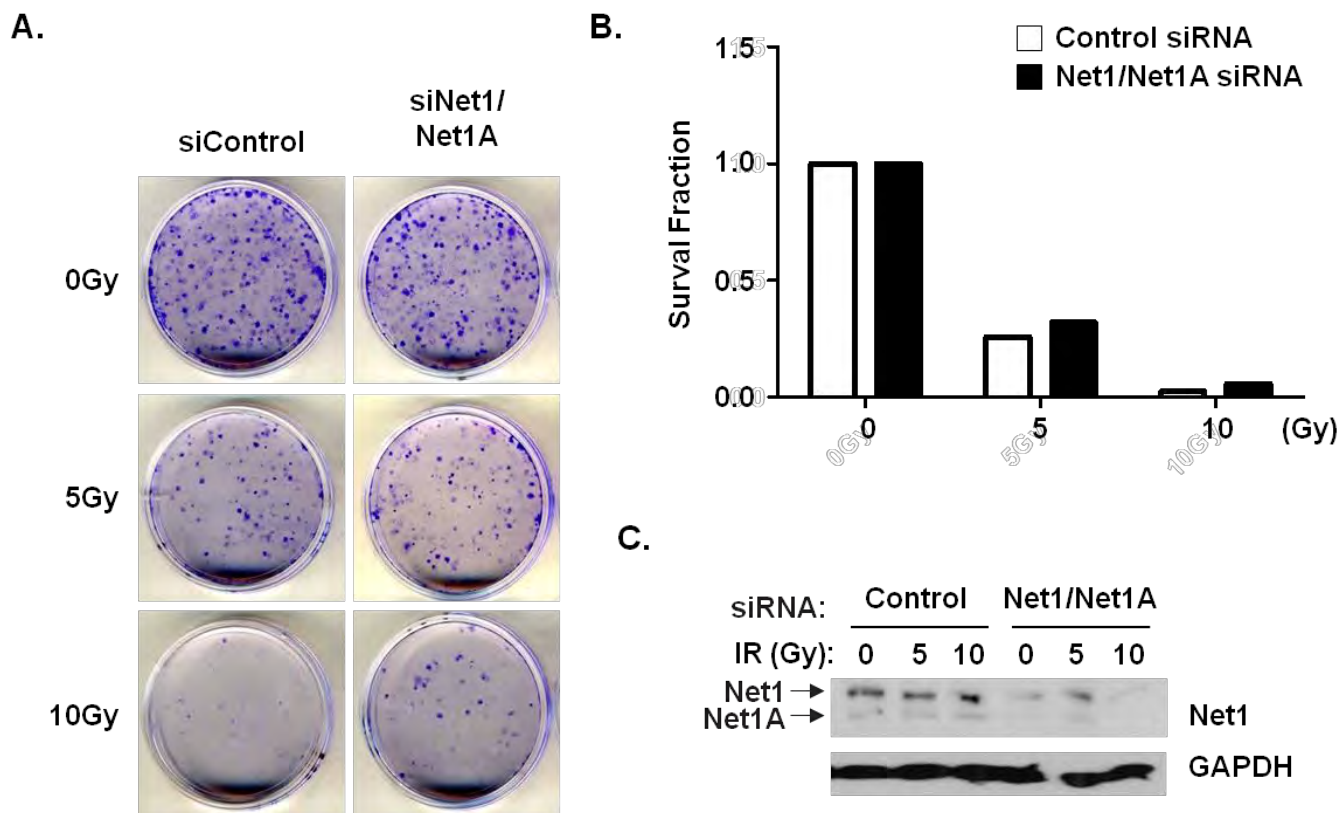


Figure 4. Net1 is not required for cell survival in normal epithelial breast cells after IR. (A) MCF12A cells were transfected with control siRNA or an siRNA specific for both Net1 isoforms. The cells were irradiated, replated and allowed to form colonies. (B) Quantification of survival fraction after IR. (C) Representative Western blot of siRNA transfected cells.

Key Research Accomplishments

- ☐ I determined that Net1A expression is required for DNA repair before and after DNA damage in breast cancer cells.
- ☐ I determined that Net1 isoforms were not required for IR-induced DNA damage signaling in normal epithelial breast cells.
- ☐ I determined that Net1 isoforms were not required for cell survival in response to IR in normal epithelial breast cells.

Reportable Outcomes

Wonkyung Oh, Jeffrey A. Frost (2014) Rho GTPase independent regulation of ATM activation and cell survival by the RhoGEF Net1A. *Cell cycle*, 13: 17, 2765-2772. (published, see Appendix 1).

Eun Hyeon Song, **Wonkyung Oh**, Heather S. Carr, Yan Zuo, and Jeffrey A. Frost (2015) Acetylation of the RhoA GEF Net1A controls its subcellular localization and activity. *J Cell Sci.*, 128: 913–922 (published, see Appendix 2).

Yan Zuo, **Wonkyung Oh**, Jeffrey A. Frost (2015) Controlling the switches: Rho GTPase regulation during animal cell mitosis. *Cell signaling*, 26: 2998-3006 (published, see Appendix 3).

Conclusions

In our published studies Net1 expression is required for cell survival in response to DNA damage. However, there has been no molecular mechanism describing how Net1 regulates DNA damage responses.

In the first year of this fellowship, a major finding of these studies was that Net1A plays an important role in controlling the activation of ATM in response to IR, as well as subsequent activation of Chk2 and H2AX. A second key finding of this study was that Net1A overexpression inhibits IR-induced DNA damage foci formation independent of its ability to stimulate the activity of its substrates RhoA or RhoB. This suggests that Net1A expression controls recruitment of PIKKs to IRIF after IR treatment. A third key finding of this study was that Net1A associates with the DNA-PK complex, which is a primary regulator of NHEJ type DNA repair after IR. Lastly, we demonstrated that Net1A depletion reduced cell survival after IR exposure. Together, these findings support a role for Net1A in DNA damage signaling and repair.

In the second year of this fellowship, we showed that the Net1A isoform did not associate with the MRN complex or MDC1, which are downstream substrates of ATM following IR exposure. Furthermore, we did not detect the effect of Net1A knockdown or overexpression on recruitment of the MRN complex or MDC1 to IRIF following IR treatment. Interestingly, Net1A overexpression inhibits IR-induced DNA damage foci formation independent of its ability to stimulate the activity of its substrates RhoA or RhoB. Thus, we found that Net1A affects ATM activation but is selective in the downstream signaling pathways that it impacts. A second key finding of this study was that Net1A knockdown reduced cell survival in breast cancer cells after IR exposure. This suggests that Net1A expression is important for cell survival in response to IR.

In the third year of this fellowship, we wanted to study the role of Net1 regulation of DNA damage responses, and investigate whether there are significant differences between normal breast epithelial cells and breast cancer cells. We showed that Net1 isoforms did not affect on the phosphorylation of ATM and its downstream substrates Chk2 and H2AX following IR exposure in normal breast epithelial cells. Moreover, Net1 did not affect IR-induced cell survival in normal breast epithelial cells, suggests that Net1 isoforms does not required for cell survival in response to IR. Thus, Net1 is required for survival after IR in breast cancer cells, but not in normal breast epithelial cells. This suggests that inhibition of Net1 function may be a useful therapeutic strategy in breast cancer. Future work will be required to identify mechanisms controlling Net1 function in DNA damage signaling, as these may provide novel avenues to inhibit Net1 function in breast cancers.

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Appendix

Appendix 1:

Rho GTPase independent regulation of ATM activation and cell survival by the RhoGEF Net1A

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ATM activation following DNA damage is a critical event which is required for efficient DNA repair and cell survival, yet signaling mechanisms controlling its activation are incompletely understood. The RhoGEF Net1 has previously been reported to control Rho GTPase activation and downstream cell survival outcomes following double strand DNA damage. However, the role of Net1 isoforms in controlling ATM-dependent cell signaling has not been assessed. In the present work we show that expression of the Net1A isoform is specifically required for efficient activation of ATM, but not the related kinase DNA-PK, after ionizing radiation. Surprisingly, Net1A overexpression also potently suppresses ATM activation and phosphorylation of its substrate H2AX. This effect does not require catalytic activity towards RhoA or RhoB, and neither Rho GTPase affects ATM activation on its own. Consistent with a role in controlling ATM activation, Net1A knockdown also impairs DNA repair and cell survival. Taken together, these data indicate that Net1A plays a previously unrecognized, Rho GTPase-independent role in controlling ATM activity and downstream signaling after DNA damage to impact cell survival.

Appendix 2:

Acetylation of the RhoA GEF Net1A controls its subcellular localization and activity

Net1 isoform A (Net1A) is a RhoA GEF that is required for cell motility and invasion in multiple cancers. Nuclear localization of Net1A negatively regulates its activity, and we have recently shown that Rac1 stimulates Net1A relocalization to the plasma membrane to promote RhoA activation and cytoskeletal reorganization. However, mechanisms controlling the subcellular localization of Net1A are not well understood. Here, we show that Net1A contains two nuclear localization signal (NLS) sequences within its Nterminus and that residues surrounding the second NLS sequence are acetylated. Treatment of cells with deacetylase inhibitors or expression of active Rac1 promotes Net1A acetylation. Deacetylase inhibition is sufficient for Net1A relocalization outside the nucleus, and replacement of the N-terminal acetylation sites with arginine residues prevents cytoplasmic accumulation of Net1A caused by deacetylase inhibition or EGF stimulation. By contrast, replacement of these sites with glutamine residues is sufficient for Net1A relocalization, RhoA activation and downstream signaling. Moreover, the N-terminal acetylation sites are required for rescue of F-actin accumulation and focal adhesion maturation in Net1 knockout MEFs. These data indicate that Net1A acetylation regulates its subcellular localization to impact on RhoA activity and actin cytoskeletal organization.

Appendix 3:

Review

Controlling the switches: Rho GTPase regulation during animal cell mitosis

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Animal cell division is a fundamental process that requires complex changes in cytoskeletal organization and function. Aberrant cell division often has disastrous consequences for the cell and can lead to cell senescence, neoplastic transformation or death. As important regulators of the actin cytoskeleton, Rho GTPases play major roles in regulating many aspects of mitosis and cytokinesis. These include centrosome duplication and separation, generation of cortical rigidity, microtubule–kinetochore stabilization, cleavage furrow formation, contractile ring formation and constriction, and abscission. The ability of Rho proteins to function as regulators of cell division depends on their ability to cycle between their active, GTP-bound and inactive, GDP-bound states. However, Rho proteins are inherently inefficient at fulfilling this cycle and require the actions of regulatory proteins that enhance GTP binding (RhoGEFs), stimulate GTPase activity (RhoGAPs), and sequester inactive Rho proteins in the cytosol (RhoGDIs). The roles of these regulatory proteins in controlling cell division are an area of active investigation. In this review we will delineate the current state of knowledge of how specific RhoGEFs, RhoGAPs and RhoGDIs control mitosis and cytokinesis, and highlight the mechanisms by which their functions are controlled.